UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF MASSACHUSETTS

ASSOCIATES OF CAPE COD, INC., AND ERIK J. PAUS

Plaintiffs,

v.

MARINE BIOLOGICAL LABORATORY, INC., AND NORMAN R. WAINWRIGHT,

Defendants.

Civil Action No. 00 CV 11275 NG

Demand for Jury Trial

COMPLAINT

Plaintiffs, Associates of Cape Cod, Inc. and Erik J. Paus (collectively "plaintiffs"), for their Complaint against defendants, Marine Biological Laboratory, and Norman R. Wainwright (collectively "defendants"), allege, upon personal knowledge as to their own acts, and upon information and belief as to all other matters, as follows:

I. The Parties

1. Plaintiff Associates of Cape Cod, Inc. ("ACC") is a corporation organized and existing under the laws of the Commonwealth of Massachusetts, with an office and a place of business at 704 Main Street, Falmouth, MA 02540. ACC is a biopharmaceutical company and is in the business of research, development, and commercialization of diagnostic and therapeutic products derived from the horseshoe crab. In particular, ACC is a manufacturer of Limulus Amebocyte Lysate ("LAL") reagent, which is widely used in the biomedical industry to detect the presence of toxins pathogenic to humans in injectable drugs and implantable medical devices. LAL reagent is prepared from the circulating blood cells of horseshoe crabs.

2. Plaintiff Erik J. Paus ("Mr. Paus") is an individual adult citizen of the Commonwealth of Massachusetts, with a residence at 740 Tubman Road, Brewster, MA 02631. Mr. Paus has been at all times relevant to this action and is currently employed as a research scientist at ACC.

3. Defendant Norman R. Wainwright ("Dr. Wainwright") is an individual adult citizen of the Commonwealth of Massachusetts, with a residence at 152 Two Ponds Road, Falmouth MA 02540. Dr. Wainwright is a former employee of ACC.

4. Defendant Marine Biological Laboratory ("MBL") is corporation organized and existing under the laws of the Commonwealth of Massachusetts, with a principal place of business at 7 Marine Biological Laboratory Street, Woods Hole, MA 02543. MBL has been and is Dr. Wainwright's current employer since his departure from ACC.

II. Jurisdiction and Venue

5. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331, 1338, and 1367.

6. Venue is proper in this judicial district pursuant to 28 U.S.C. § 1391.

7. Jurisdiction over defendants comports with the United States Constitution and federal statutes, and the laws of the Commonwealth of Massachusetts.

III. Nature of the Action

8. This is an action to correct inventorship of United States Patent No. 5,780,429 ("the '429 patent") (attached hereto as Ex. A) pursuant to 35 U.S.C. § 256 and for breach of contract, breach of a confidentiality agreement, breach of a confidential relationship, fraud,

unjust enrichment, intentional interference with an advantageous business relationship, misappropriation of trade secrets in violation of M.G.L. c. 93, §42, and unfair competition in violation of M.G.L. c. 93A, §11. This is also an action for a declaratory judgment adjudging Mr. Paus a co-inventor of the '429 patent, and ACC the owner of the '429 patent.

9. All of the above causes of action arise out of defendants' misappropriation of ACC's confidential and proprietary information. Defendants misappropriated ACC's confidential and proprietary scientific research relating to certain compositions derived from the horseshoe crab, and improperly used that information, in part, to obtain the '429 patent. Also, the '429 patent fails to identify Mr. Paus as a co-inventor of the subject matter claimed in that patent.

IV. Factual Allegations

10. On June 1, 1986, Dr. Wainwright started his employment with ACC as a research scientist.

11. On June 6, 1986, Dr. Wainwright executed a letter agreement with ACC (the "1986 Letter Agreement"), which provides, in relevant part, as follows (attached hereto as Exhibit B):

> I hereby grant to you all of my rights to patents . . . or other benefits of whatever nature resulting from research in which I engage as your employee and will assist you at all times in every proper way . . . to obtain for your benefit such patents. . . .
>
> I will not at any time reveal, divulge or make known to anyone any information or knowledge which I received or acquire[d] as a result[] of research in which I engage as your employee.

12. At ACC, Dr. Wainwright served as Director of Research. In this position, he had responsibility for developing new products derived from the horseshoe crab. More specifically, his work related to the isolation, purification and characterization of substances derived from the horseshoe crab that have therapeutic, diagnostic and other applications. Endotoxin Neutralizing Protein ("ENP"), also known as anti-LPS factor, is one of the various such substances upon which Dr. Wainwright's work at ACC focused. ENP neutralizes endotoxins, substances that under certain circumstances can be harmful to mammals. ENP neutralizes by binding to the endotoxin. This significantly reduces or abolishes the endotoxin's ability to induce inflammatory responses in mammals.

13. At ACC, Dr. Wainwright had responsibility for investigating all aspects of ENP and for developing a product using this technology that could be commercialized. Consequently, during Dr. Wainwright's tenure, ACC had a vigorous research program relating to ENP.

14. In the Spring of 1990, Mr. Paus, one of Dr. Wainwright's colleagues at ACC, conducted experiments to determine the antibiotic effects of ENP, specifically, to see if a synergy exists between ENP and other antibiotics. Mr. Paus' experiments showed that such a synergy existed. That is, Mr. Paus' work established that the combined effect of ENP with other antibiotics was not merely additive, but multiplicative, and thus, synergistic. This discovery was surprising.

15. Mr. Paus communicated the results of these experiments to Dr. Wainwright prior to Dr. Wainwright's departure from ACC.

16. On December 12, 1991, Dr. Wainwright executed an agreement that confirmed that the above provisions of the 1986 Letter Agreement would continue in effect after the termination of his employment with ACC.

17. On December 31, 1991, Dr. Wainwright's employment with ACC ended.

18. Thereafter, from January 1, 1992 to approximately February 24, 1992, Dr. Wainwright performed "research on ACC's behalf" as an independent contractor. This work concerned the same subject matter as Dr. Wainwright's work while an employee of ACC, and was subject to the provisions of the 1986 Letter Agreement.

19. On or about March 1992, Dr. Wainwright became an employee of MBL.

20. After joining MBL, Dr. Wainwright disclosed to MBL information concerning confidential and proprietary scientific research conducted at ACC relating to ENP, including, but not limited to, Mr. Paus' discovery that ENP had a synergistic effect when combined with other antibiotics.

21. At the time Dr. Wainwright divulged it to MBL, that information had not been publically disclosed by ACC and ACC had taken reasonable steps to maintain the confidentiality of that information. Such information constituted a trade secret (hereinafter "ACC's Trade Secrets").

22. On December 22, 1995, Dr. Wainwright, with the knowledge and encouragement of MBL, filed with the United States Patent and Trademark Office ("PTO"), patent application number 08/577,464 ("the '464 Application"). The '464 Application contained ACC's Trade Secrets and, in particular, was based upon and, at least in part, derived from Mr. Paus' work at ACC concerning the synergistic effect of ENP.

23. Defendants filed the '464 Application without ACC's knowledge or consent.

24. The '464 Application failed to name Mr. Paus as a co-inventor. Mr. Paus is in fact a true co-inventor of the subject matter claimed in that application.

25. On July 14, 1998, the PTO issued the '429 patent, which is based upon the '464 Application. The '429 patent is entitled, "Anti-LPS Factor From Horseshoe Crabs and Methods of Use."

26. The '429 patent relates to therapeutic methods of preventing or treating mircrobial infections by administering ENP or ENP-derivatives alone or in combination with antibiotics. The '429 patent also relates to pharmaceutical compositions comprising ENP or ENP-derivatives that can be used in connection with such methods.

27. The specification of the '429 patent states that Dr. Wainwright discovered that ENP from horseshoe crabs produces a synergistic effect when combined with antibiotics. This contention is false as Mr. Paus actually discovered this effect at ACC and then shared his discovery with Dr. Wainwright, who at the time was Mr. Paus' supervisor at ACC. Thus, the '429 patent's listing of Dr. Wainwright as the sole inventor of the '429 patent is erroneous. Mr. Paus is a true co-inventor of the '429 patent and should have been so listed. As Mr. Paus' assignee, ACC is, at least, a true co-owner of the '429 patent.

28. The '429 patent identifies MBL as the sole assignee. On information and belief, Dr. Wainwright assigned all rights in the '429 patent solely to MBL. Dr. Wainwright's assignment of all of his rights and interests in the '429 patent solely to MBL is unlawful. The inventions claimed in the '429 patent arose, at least in part, from Dr. Wainwright's work at ACC. Accordingly, ACC is at least a co-owner of the '429 patent. Consequently, pursuant to the 1986 Letter Agreement, Dr. Wainwright should have assigned all of his rights in the '429 patent to ACC that are based upon and, at least in part, derived from his work at ACC. He violated the 1986 Letter Agreement when he did not do so.

29. On information and belief, MBL had knowledge of, conspired in, or adopted Dr. Wainwright's wrongful conduct.

30. Despite MBL's knowledge that the '429 patent is based upon and derived from confidential and proprietary information belonging to ACC, specifically, ACC's Trade Secrets, MBL continues to use that information for its own purposes and to the determent of ACC.

31. MBL has refused and continues to refuse to relinquish its improper sole ownership of the '429 patent.

V. Claims for Relief

Count One

Correction of Inventorship Under 35 U.S.C. § 256

32. Plaintiffs incorporate the allegations of paragraphs 1 through 31 above.

33. Mr. Paus is entitled to be listed as a named co-inventor of the '429 patent.

34. Without any deceptive intent by Mr. Paus, he was omitted as a named inventor of the '429 patent.

35. Plaintiffs are entitled to an order of this Court correcting inventorship of the '429 patent whereby Mr. Paus is named as a co-inventor along with defendant Dr. Wainwright.

Count Two

Breach of Contract
(Dr. Wainwright Only)

36. Plaintiffs incorporate the allegations of paragraphs 1 through 35 above.

37. As set forth above, ACC and Dr. Wainwright entered into an agreement that required Dr. Wainwright to assign to ACC all inventions of whatever nature resulting from research during his tenure at ACC ("the Assignment Agreement").

38. ACC duly performed all of its obligations, conditions, and covenants under the Assignment Agreement.

39. Dr. Wainwright, by the wrongful acts described above, has materially breached the Assignment Agreement by failing to assign to ACC his interests in the '429 patent.

40. ACC has suffered irreparable injury as a result of Dr. Wainwright's wrongful conduct, has no adequate remedy at law, and is entitled to the injunctive relief set forth in this Complaint.

41. ACC has suffered damages arising out of Dr. Wainwright's wrongful conduct in amount that has yet to be determined but which will be proved at trial.

Count Three

Breach of Confidentiality Agreement
(Dr. Wainwright Only)

42. Plaintiffs incorporate the allegations of paragraphs 1 through 41 above.

43. As set forth above, ACC and Dr. Wainwright entered into an agreement that required Dr. Wainwright to not reveal, divulge or make known to anyone any information or knowledge that he received or acquired as a result of research during his tenure at ACC ("the Confidentiality Agreement").

44. ACC duly performed all of its obligations, conditions, and covenants under the Confidentiality Agreement.

45. Dr. Wainwright, by the wrongful acts described above, has materially breached the Confidentiality Agreement by failing to keep Mr. Paus' research and other scientific research conducted at ACC confidential. Specifically, without ACC's consent, Dr. Wainwright has disclosed ACC's confidential information to MBL and has used such information as the basis for obtaining the '429 patent.

46. ACC has suffered irreparable injury as a result of Dr. Wainwright's wrongful conduct, has no adequate remedy at law, and is entitled to the injunctive relief set forth in this Complaint.

47. ACC has suffered damages arising out of Dr. Wainwright's wrongful conduct in an amount that has yet to be determined but which will be proved at trial.

Count Four

Breach of a Confidential Relationship
(Dr. Wainwright Only)

48. Plaintiffs incorporate the allegations of paragraphs 1 through 47 above.

49. ACC provided Dr. Wainwright access to materials and information concerning or reflecting scientific research conducted at ACC for the sole purpose of allowing Dr. Wainwright to perform his responsibilities as an employee of ACC. ACC indicated that such materials and information were proprietary and strictly confidential, and ACC fully and reasonably expected that Dr. Wainwright would keep such information and materials confidential.

50. Dr. Wainwright understood that ACC's confidential and proprietary materials and information were not to be disclosed to others and Dr. Wainwright was not to use the materials and/or information for his own purposes.

51. Dr. Wainwright breached his confidential relationship with ACC by disclosing ACC's confidential and proprietary materials and information to others, including MBL and in the patent application that lead to the '429 patent.

52. Unless enjoined Dr. Wainwright will continue to disclose and wrongfully utilize ACC's confidential and proprietary information in breach of his confidential relationship with ACC.

53. ACC has suffered irreparable injury as a result of Dr. Wainwright's wrongful conduct for which there is no adequate remedy at law.

54. ACC has suffered damages arising out of Dr. Wainwright's wrongful conduct in an amount that has yet to be determined but which will be proved at trial.

Count Five

Fraud
(Dr. Wainwright Only)

55. Plaintiffs' incorporate the allegations of paragraphs 1 through 54 above.

56. On or about July 6, 1986, Dr. Wainwright represented to ACC that he would not disclose ACC's confidential and proprietary information to third parties without ACC's consent.

57. On or about December 12, 1991, Dr. Wainwright again represented to ACC that its confidential and proprietary information would be kept confidential.

58. When Dr. Wainwright made these representations, he knew them to be false and made them with the intent to deceive ACC and to induce it to act in reliance on these representations by providing him access to confidential and proprietary information and to provide him financial remuneration.

59. At the time these representations were made by Dr. Wainwright, ACC reasonably believed that they were true, and in reasonable reliance on these representations, ACC was induced and did provide Dr. Wainwright access to ACC's confidential and proprietary information, and financial remuneration. Had ACC known the actual facts, it would not have taken such action.

60. As a proximate result of the fraudulent conduct of Dr. Wainwright, ACC has suffered irreparable injury for which there is no adequate remedy at law.

61. As a proximate result of the fraudulent conduct of Dr. Wainwright, ACC has suffered damages in an amount that has yet to be determined but which will be proved at trial.

Count Six

Unjust Enrichment

62. Plaintiffs incorporate the allegations of paragraphs 1 through 61 above.

63. Defendants have received a benefit by virtue of their improper acquisition of all rights, title and interest in the '429 patent.

64. Defendants have accepted and retained the benefits derived from their improper acquisition of all rights, title and interest in the '429 patent under circumstances that make it inequitable for defendants to retain these befits with paying for them.

65. Defendants have full knowledge and appreciation of receiving the benefits derived from their improper acquisition of all rights, title and interest in the '429 patent. However, defendants have refused to pay plaintiffs the value of the benefits received from those rights.

66. By reason of defendants' improper acquisition of all rights, title and interest in the '429 patent without making payment to plaintiffs, plaintiffs have sustained damages in an amount to be determined at trial for the value of the benefits conferred upon each defendant.

Count Seven

Intentional Interference with Advantageous Business Relationship
(MBL Only)

67. Plaintiffs incorporate the allegations of paragraphs 1 through 66 above.

68. MBL had knowledge of Dr. Wainwright's legal obligations pursuant to his Letter Agreement with ACC.

69. MBL induced and encouraged and assisted Dr. Wainwright in breaching his 1986 Letter Agreement thereby interfering with that agreement, improperly obtaining sole ownership of the '429 patent, and causing irreparable harm to ACC for which there is no adequate remedy at law.

70. MBL acted willfully and maliciously in interfering with the contractual relationship between Dr. Wainwright and ACC.

71. ACC has suffered damages arising out of MBL's intentional interference with Dr. Wainwright's performance of his obligations under the 1986 Letter Agreement. The amount of damages has not yet been ascertained but will be proved at trial.

Count Eight

Misappropriation of Trade Secrets
(Violation of M. G. L. c. 93 § 42)

72. Plaintiffs incorporate the allegations of paragraphs 1 through 71 above.

73. As described above, Dr. Wainwright disclosed ACC's Trade Secrets to MBL, and defendants then included those trade secrets in the '429 patent without ACC's knowledge or consent.

74. ACC took reasonable steps to keep its Trade Secrets confidential. Such steps included but are not limited to requiring its employees, including Dr. Wainwright, to sign confidentiality agreements obligating them to not disclose such information to third parties without ACC's consent.

75. ACC's Trade Secrets have been researched and developed over a long period of time and required the expenditure of substantial money, expertise and effort.

76. Dr. Wainwright was obligated to maintain the secrecy of ACC's Trade Secrets as a matter of law and pursuant to the terms of his non-disclosure agreement.

77. Defendants have misappropriated ACC's Trade Secrets and utilized such information to obtain the '429 patent.

78. Defendants have improperly disclosed ACC's Trade Secrets by using them as the basis for obtaining the '429 patent.

79. Defendants' acquisition and exploitation of the '429 patent and their use of ACC's Trade Secrets results from the use of improper means to acquire ACC's Trade Secrets and Dr. Wainwright's breach of his confidential relationship. MBL is aware that Dr. Wainwright has breached such relationship, and that MBL's purported sole ownership of the '429 patent arose from that breach.

80. Defendants conduct as described herein is a violation of M. G. L. c. 93 §42.

81. Unless enjoined, defendants will continue to use and disclose ACC's Trade Secrets.

82. Defendants' use and disclosure of ACC's Trade Secrets will irreparably damage ACC by damaging the position it enjoys by virtue of its Trade Secrets and by depriving ACC of the benfits to which it is entitled as a result of the expenditures of substantial amounts of time and money in developing ACC's Trade Secrets.

83. ACC has no adequate remedy at law for the injuries currently being suffered and therefore ACC is entitled to the injunctive relief requested in this Complaint.

84. ACC has suffered actual losses and is therefore entitled to an award of damages equal to its actual losses as a result of defendants' wrongful conduct. Alternatively, ACC is entitled to a sum equal to the amount by which defendants have been unjustly enriched as a result of their misappropriation of ACC's Trade Secrets.

Count Nine

Unfair Competition
(Violation of M.G.L. c. 93A, §11)

85. Plaintiffs incorporate the allegations of paragraphs 1 through 84 above.

86. By the acts described above, defendants have engaged in unfair competition, and/or unlawful, deceptive and misleading business practices.

87. Defendants' wrongful conduct occurred within the Commonwealth of Massachusetts.

88. Defendants' practices and acts have injured ACC in its business and property and entitle it to compensatory damages, punitive damages and an injunction restraining defendants from conducting their unfair business practices.

Count Ten

Declaratory Judgment-Constructive Trust

89. Plaintiffs incorporate the allegations of paragraphs 1 through 88 above.

90. By reason of the allegations herein, MBL must be deemed to hold the '429 patent and all rights that derive therefrom constructively in trust for the benefit of ACC, the rightful and true owner of the '429 patent by assignment from co-inventors Dr. Wainwright and Mr. Paus.

91. Mr Paus is entitled to a declaration adjudging that he is a true co-inventor of the '429 patent.

92. ACC, as Dr. Wainwright's and Mr. Paus' assignee, is entitled to a declaration adjudging that it is the owner of all legal rights, title and interests to the '429 patent and any and all rights that derive therefrom.

WHEREFORE, plaintiffs demand judgment against defendants granting to them:

1. With respect to Count One:

   (a) For an Order directing the Commissioner of Patents and Trademarks to issue a certificate correcting inventorship of United States Patent No. 5,780,429 to add Erik J. Paus as inventor.

2. With respect to Count Two:

   (a) For an order voiding Dr. Wainwright's assignment of all rights, title and interests in United States Patent No. 5,780,429 to MBL; and

   (b) For an order directing Dr. Wainwright to assign all of his rights, title and interests in United States Patent No. 5,780,429 to ACC as an owner; and

   (b) For compensatory damages in an amount to be proved at trial.

3. With Respect to Count Three

(a) For an injunction permanently enjoining Dr. Wainwright and all individuals, firms, corporations, associations, or partnerships affiliated, associated, or acting in concert with him in any way, including MBL:

(1) From disclosing in any manner ACC's confidential information; and

(2) From utilizing or using in any manner ACC's confidential information.

(b) For compensatory damages in an amount to be proved at trial.

4. With respect to Count Four

(a) For an injunction permanently enjoining Dr. Wainwright and all individuals, firms, corporations, associations, or partnerships affiliated, associated, or acting in concert with him in any way, including MBL:

(1) From disclosing in any manner ACC's confidential information; and

(2) From utilizing or using in any manner ACC's confidential information.

(b) For compensatory damages in an amount to be proved at trial; and

(c) For punitive damages in an amount to be proved at trial.

5. With respect to Count Five:

(a) For an injunction permanently enjoining Dr. Wainwright and all individuals, firms, corporations, associations, or partnerships affiliated, associated, or acting in concert with him in any way, including MBL:

(1) From disclosing in any manner ACC's confidential information; and

(2) From utilizing or using in any manner ACC's confidential information.

(b) For compensatory damages in an amount to be proved at trial; and

(c) For punitive damages in an amount to be proved at trial.

6. With respect to Count Six:

(a) For an award of compensatory damages in an amount to be determined at trial for the value of the benefits conferred upon the defendants.

7. With respect to Count Seven:

(a) For an injunction permanently enjoining MBL and all individuals, firms, corporations, associations, or partnerships affiliated, associated, or acting in concert with him in any way, including Dr. Wainwright:

(1) From disclosing in any manner ACC's confidential information; and

(2) From utilizing or using in any manner ACC's confidential information.

(b) For compensatory damages in an amount to be proved at trial; and

(c) For punitive damages in an amount to be proved at trial.

8. With respect to Count Eight:

(a) For an injunction permanently enjoining MBL and all individuals, firms, corporations, associations, or partnerships affiliated, associated, or acting in concert with him in any way, including Dr. Wainwright:

(1) From disclosing in any manner ACC's confidential information; and

(2) From utilizing or using in any manner ACC's confidential information.

(b) For compensatory damages in an amount to be proved at trial; and

(c) For punitive damages in an amount to be proved at trial.

9. With respect to Count Nine:

(a) For an injunction permanently enjoining MBL and all individuals, firms, corporations, associations, or partnerships affiliated, associated, or acting in concert with him in any way, including Dr. Wainwright:

(1) From disclosing in any manner ACC's confidential information; and

(2) From utilizing or using in any manner ACC's confidential information.

(b) For compensatory damages in an amount to be proved at trial; and

(c) For punitive damages in an amount to be proved at trial.

10. With respect to Count Ten:

(a) A declaration adjudging that Mr. Paus is a true co-inventor of the '429 patent;

(b) A declaration adjudging that ACC, as Dr. Wainwright's and Mr. Paus' assignee, is the true owner of all legal rights, title and interests to the '429 patent and any and all rights that derive therefrom; and

(c) A declaration that for such time as any defendant was the holder of any legal title in the '429 patent, that defendant held such property rights in constructive trust for the benefit of plaintiffs.

11. With respect to all Counts:

(a) For costs of suit, including attorneys' fees to the extent permitted by law; and

(b) For such other relief as the Court deems just and proper.

VI. Demand for Jury Trial

93. Plaintiffs demand trial by jury of all issues so triable in this cause.

Respectfully submitted,

Dated: June 29, 2000 By: Marc E. Jurtalis

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ERIK J. PAUS

US005780429A

United States Patent [19]

Wainwright

[11] Patent Number: 5,780,429

[45] Date of Patent: Jul. 14, 1998

[54] ANTI-LPS FACTOR FROM HORSESHOE CRABS AND METHODS OF USE

[75] Inventor: Norman R. Wainwright, Falmouth, Mass.

[73] Assignee: Marine Biological Laboratory, Woods Hole, Mass.

[21] Appl. No.: 577,464

[22] Filed: Dec. 22, 1995

[51] Int. Cl.[6] A61K 38/00

[52] U.S. Cl. 514/12; 530/350; 530/857; 424/70.1

[58] Field of Search 424/70.1; 514/12, 514/21; 530/350, 857

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Primary Examiner—Cecilia J. Tsang
Assistant Examiner—C. Delacroix-Muirheid
Attorney, Agent, or Firm—Hale and Dorr LLP

[57] ABSTRACT

This invention is directed to pharmaceutical and cosmetic compositions comprising anti-lipopolysaccharide (anti-LPS) factor proteins derived from horseshoe crabs, either in the native form or produced by recombinant means. The pharmaceutical formulations, which may include anti-LPS factor proteins alone or in combination with other [illegible] may be used in the treatment of gram-negative bacterial infections, endotoxemia, septic shock, gram-positive bacterial infections, and yeast infections. The anti-LPS factor protein-containing pharmaceuticals can be formulated for systemic or topical administration. They may also be used to control mold growth. Anti-LPS factor proteins can be used in cosmetic compositions or skin or hair preparations as antimicrobial preservatives, either alone or in combination with conventional preservatives, to prevent or control the growth of bacteria, yeast and mold.

6 Claims, 5 Drawing Sheets

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GAT GGT ATT TGG ACT CAA TTG ATT TTT ACT TTG GTT AAT AAT TTG GCT
Asp Gly Ile Trp Thr Gln Leu Ile Phe Thr Leu Val Asn Asn Leu Ala
 1               5                  10                  15

ACT TTG TGG CAA TCT GGT GAT TTT CAA TTT TTG GAT CAT GAA TGT CAT
Thr Leu Trp Gln Ser Gly Asp Phe Gln Phe Leu Asp His Glu Cys His
            20                  25                  30

TAT AGA ATT AAA CCA ACT TTT AGA AGA TTG AAA TGG AAA TAT AAA GGT
Tyr Arg Ile Lys Pro Thr Phe Arg Arg Leu Lys Trp Lys Tyr Lys Gly
        35                  40                  45

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Lys Phe Trp Cys Pro Ser Trp Thr Ser Ile Thr Gly Arg Ala Thr Lys
    50                  55                  60

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Ser Ser Arg Ser Gly Ala Val Glu His Ser Val Arg Asn Phe Val Gly
65                  70                  75                  80

CAA GCT AAA TCT TCT GGT TTG ATT ACT CAA AGA CAA GCT GAA CAA TTT
Gln Ala Lys Ser Ser Gly Leu Ile Thr Gln Arg Gln Ala Glu Gln Phe
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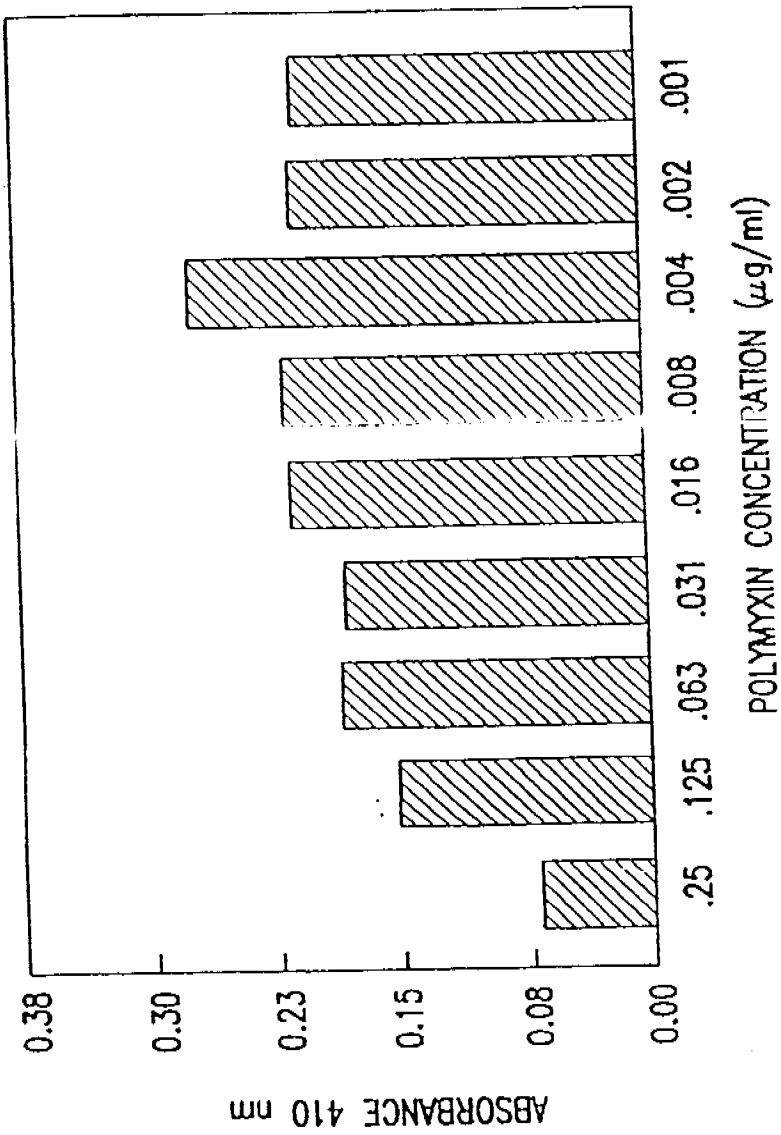
FIG.1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0.23
0.15
0.08
0.00
ABSORBANCE 410 nm
.25
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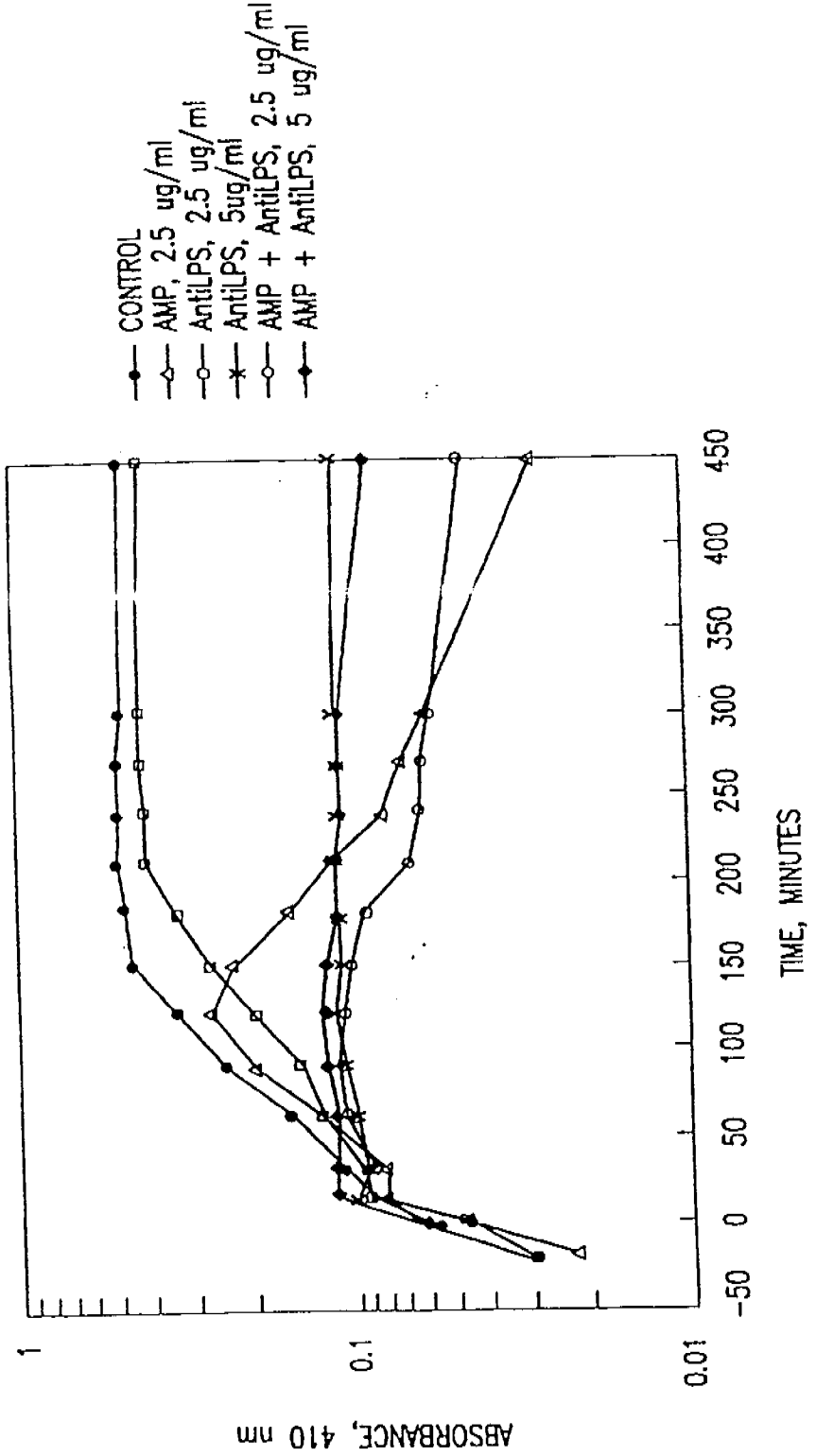
.063
.031
.016
.008
.004
.002
.001
POLYMYXIN CONCENTRATION (μg/ml)

FIG.2A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AntiLPS, 2.5 ug/ml
AntiLPS, 5ug/ml
AMP + AntiLPS, 2.5 ug/ml
AMP + AntiLPS, 5 ug/ml
1
0.1
0.01
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-50
0
50
100
150
200
250
300
350
400
450
TIME, MINUTES

FIG.3

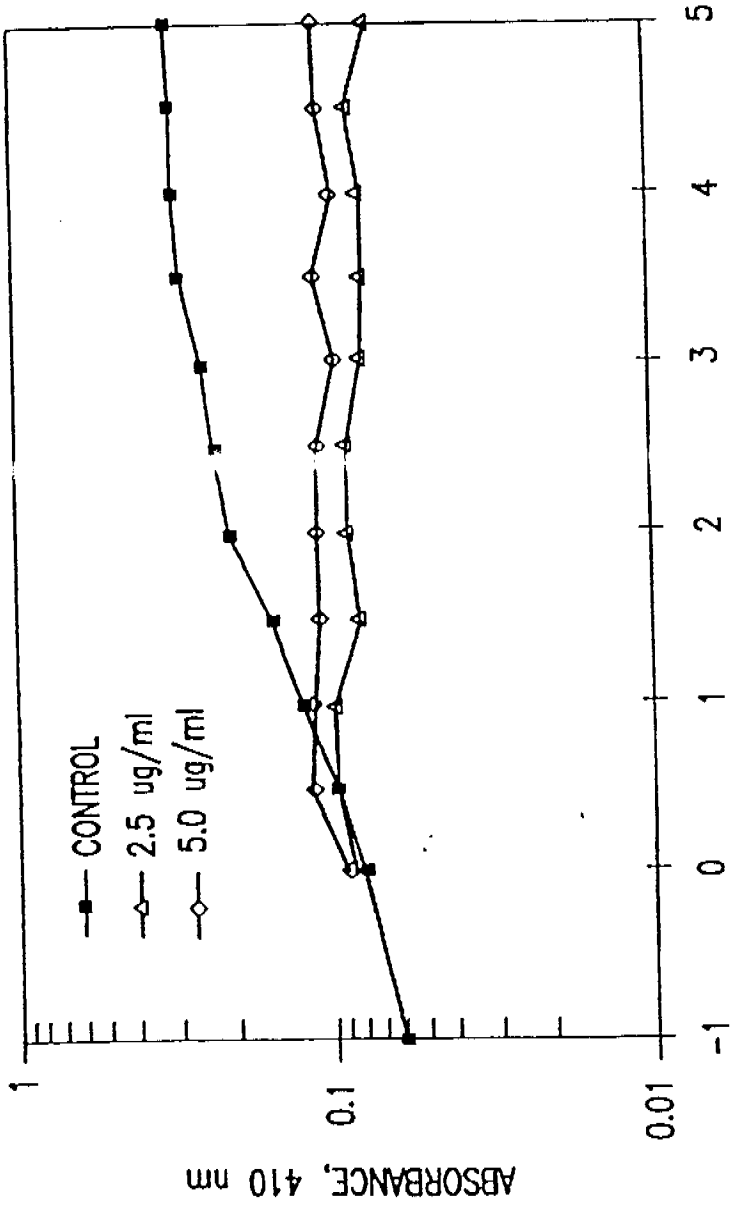
ABSORBANCE, 410 nm
1
0.1
0.01
-1
0
1
2
3
4
5
TIME, HOURS
CONTROL
2.5 ug/ml
5.0 ug/ml

FIG.4

ANTI-LPS FACTOR FROM HORSESHOE CRABS AND METHODS OF USE

FIELD OF THE INVENTION

The present invention is in the field of antibacterial pharmaceutical compositions and in the field of antimicrobial preservatives.

BACKGROUND OF THE INVENTION

Endotoxins are high molecular weight lipopolysaccharide complexes that are released when gram-negative bacteria are disrupted, such as occurs during antibiotic therapy when bacteria are lysed. Endotoxins, an outer wall constituent of the gram-negative bacteria, are potent stimulators of the inflammatory response which produce pyrogenic reactions upon intravenous administration. While an inflammatory response that is measured is beneficial in fighting infection, it can be damaging to the host when it is uncontrolled, as is the case with septic shock.

Bacterial endotoxin is a complex of lipid, carbohydrate and protein. It is characterized by an overall negative charge, heat stability and high molecular weight. Highly purified endotoxin is a lipopolysaccharide (LPS) that does not contain protein. It is the lipopolysaccharide component of bacterial endotoxins that causes endotoxemia and septic shock.

LPS consists of three distinct chemical regions: (1) the phospholipid moiety (lipid A), which is the innermost region of LPS and the source of toxicity; (2) an intermediate core polysaccharide; and (3) an outermost O-specific polysaccharide side chain which is responsible for the antigenicity of the endotoxin.

LPS from gram-negative bacteria induces the release of mediators from host inflammatory cells which may ultimately result in disseminated intravascular coagulation (DIC), adult respiratory distress syndrome (ARDS), cardiac dysfunction, organ failure, liver failure (hepatobiliary dysfunction), brain failure (CNS dysfunction), renal failure, multi-organ failure and shock.

While there are many antibiotics used against gram-negative bacterial infections, due to the increase in antibiotic resistant bacteria, there is still a need to identify effective antibiotics.

SUMMARY OF THE INVENTION

This invention is directed to pharmaceutical compositions and uses of anti-lipopolysaccharide (anti-LPS) factor proteins derived from horseshoe crabs, either in the native form or produced by recombinant or synthetic means. The pharmaceutical formulations may be used in the treatment of gram-negative bacterial infections, endotoxemia and septic shock. The invention is further directed to pharmaceutical compositions and uses of anti-LPS factor proteins against infections by gram-positive bacteria. The invention is also directed to pharmaceutical compositions and uses of anti-LPS factor proteins as an antimycotic agent. The pharmaceutical compositions may include anti-LPS factor proteins alone or in combination with other known antibiotics or antimycotic agents.

Finally, this invention is also directed to the use of anti-LPS factor proteins as antimicrobial preservatives, either alone or in combination with conventional preservatives, in cosmetics or skin or hair preparations to prevent or control the growth of bacteria, yeast and mold.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is the nucleotide sequence (SEQ. ID NO:1) for the gene encoding LALF, Limulus anti-LPS factor, with the derived amino acid sequence (SEQ. ID NO:2).

FIG. 2(A) is a graphic representation of the inhibitory effect of increasing concentrations of the antibiotic polymyxin B (PMB) on the growth of the gram-negative bacteria *E. coli*. FIG. 2(B) is a graphic comparison of the inhibitory effect of increasing concentrations of LALF on the growth of *E. coli* with the effect of a combination antibiotic containing 0.06 micrograms/ml polymyxin B and increasing concentrations of LALF.

FIG. 3 is a graphic comparison of the inhibitory effects on the growth of *E. coli* of (1) 2.5 micrograms/ml ampicillin, (2) 2.5 micrograms/ml LALF, (3) 5.0 micrograms/ml LALF, (4) 2.5 micrograms/ml ampicillin plus 2.5 micrograms/ml LALF, (5) and 2.5 micrograms/ml ampicillin plus 5 micrograms/ml LALF.

FIG. 4 is a graphic representation of the inhibitory effect of 2.5 and 5 micrograms/ml LALF on uncharacterized gram-positive bacteria isolated from marine organisms.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed, in part, to the use of anti-LPS factor proteins in the preparation of new and more potent antibiotics for treating bacterial and yeast infections. It has been discovered that anti-LPS factor proteins from horseshoe crabs produce a synergism in controlling gram-negative bacterial infections when combined with antibiotics that are known to be effective against gram-negative infections. New antibiotics for treating gram-negative bacterial infections are therefore made by combining anti-LPS factor proteins with antibiotics that are known to be effective in controlling gram-negative bacterial infections.

It has also been discovered that anti-LPS factor proteins are effective antibiotics for treating gram-positive bacterial infections and yeast infections. Anti-LPS factor proteins also control the growth of mold. In addition to being effective against these microbes when administered alone, anti-LPS factor proteins can also be combined with one or more known antimicrobials that are effective against gram-positive bacteria, yeast and mold.

The new anti-LPS factor protein-based therapeutics of the present invention can be formulated for systemic or topical administration.

Finally, anti-LPS factor proteins are useful antimicrobial preservatives, either alone or in combination with conventional preservatives, in cosmetics or skin and hair preparations where they prevent and control the growth of a broad spectrum of bacteria, yeast and mold for extended periods of time.

I. Definitions.

As used in this application, the following words or phrases have the meanings specified:

"LPS" means lipopolysaccharide which is used synonymously with the word "endotoxin."

"Anti-LPS factor" means a protein isolated from any species of horseshoe crab, that is capable of binding to and neutralizing the biological activity of bacterial endotoxins.

"Anti-LPS factor protein" means (1) anti-LPS factor derived from any species of horseshoe crab; (2) biologically active fragments of anti-LPS factor; or (3) biologically active polypeptide analogs of anti-LPS factor, each of which may be either recombinant, nonrecombinant, or synthetic.

"Synthetic analogs means any polypeptide which has substantially the same amino acid sequence as anti-LPS factor and is chemically synthesized.

"Endotoxin" means a toxin from a gram-negative bacterium which is pyrogenic.

II. Anti-lipopolysaccharide (anti-LPS) factor proteins.

Anti-LPS factor proteins for use in the present invention may be isolated from amebocytes of any horseshoe crab. For example, any of the four known species of horseshoe crabs could be used. These species are: *Limulus polyphemus, Tachypleus gigas, Tachypleus tridentatus* and *Carcinoscropius rotundicauda*. Especially preferred among these is *Limulus polyphemus*, the horseshoe crab which is found along the North American coast. The methods for isolating and purifying native or endogenous anti-LPS factor are well known. WO 92/20715, WO 89/12644, and Kloczewiak, M., et al., *J. Infectious Diseases*, Vol. 170, 1490–97(1994).

A preferred anti-LPS factor protein for use in this invention is the anti-LPS factor produced by *Limulus polyphemus*, called Limulus anti-LPS factor or LALF. LALF may be used in the various embodiments of this invention as a purified endogenous protein and as a recombinant protein. LALF has been isolated, sequenced and expressed as a recombinant protein in *Saccharomyces cerevisiae*, as described in Kuppermann, N., et al., *J. Infect. Dis.*, Vol 170, 630–5 (1994), and as described below in the Pichia expression system. Recombinant LALF expressed in Pichia has the same amino acid sequence as native LALF and is the preferred anti-LPS factor protein for use in the present invention. Both the gene sequence (SEQ ID NO:1) and the derived amino acid sequence (SEQ ID NO:2) for LALF transcribed from yeast preferred codons in Pichia are shown in FIG. 1.

Although LALF is a preferred anti-LPS factor protein, the anti-LPS factor proteins from the other species of horseshoe crabs, which are approximately 70% homologous to LALF, may also be used in the various embodiments of the invention.

LALF is an 11.8-kDa protein that inhibits the biologic effects of endotoxin in vitro, including the gelation of Limulus amebocyte lysate (LAL). Wainwright, N. R., et al., *In: Cellular and Molecular Aspects of Endotoxin Reactions*, Nowotny A., Spitzer, J. J. and Ziegler, E. J., eds., *Amsterdam: Elsevier Science*, 315–25 (1990).

The endotoxin neutralizing activity of anti-LPS factor proteins derives from a high affinity for the lipid A portion of endotoxin. In the horseshoe crab, anti-LPS factor is part of an anti-infection pathway of aggregation where rapid degranulation and clot formation are initiated when hematocytes containing anti-LPS factor proteins are exposed to gram-negative endotoxins.

Anti-LPS factor protein has a 16 amino acid domain that is necessary for endotoxin binding and neutralization. WO 92/20715. This domain comprises the amino acid sequence of Limulus anti-LPS factor (LALF) from amino acid position 30 to amino acid position 55. Synthetic peptide analogues of LALF have been made, and the site of the activity and specific sequence needed to bind and neutralize endotoxin have been determined. Kloczewiak, M., et al., *Journal of Infectious Diseases*, Vol. 170, 1490–97 (1994).

Anti-LPS factor proteins are single chain, basic proteins that can bind to and neutralize LPS. This amphipathic protein has a rich clustering of hydrophobic amino acids at the amino terminal region and an array of basic amino acids in the central disulfide-bonded loop region. When LPS and anti-LPS factor proteins are mixed, they aggregate. The exact mode of binding is uncertain, although it is likely that the hydrophobic and cationic amino acids of anti-LPS factor proteins interact, respectively, with the fatty acid chains and the phosphate groups of the toxic lipid A region of LPS.

The endotoxin binding phenomenon by Limulus anti-LPS factor is universal. Studies have shown that LALF binds to and inactivates endotoxin from *Klebsiella pneumonias, Serratia marcescens, Salmonella enteritidis, Escherichia coli* 0113 wild type, *E. coli* rough mutant (J-5), *Salmonella abortus equi*, and Lipid A from *S. minnesota* Re 595. WO 92/20715. In these studies, the endotoxin binding protein was mixed with the endotoxins or lipid A at a ratio of 5:1 to 1,000:1 in the presence of 10 millimolar Tris buffer, pH 6–8. In all cases the measurable endotoxin activity after mixing was reduced 85% to 99.5%.

It has also been shown that anti-LPS factor proteins inhibit endotoxin-induced mitogenesis of murine splenocytes, Warren, H. S., et al., *Infect. Immun.*, Vol. 60, 2506–13 (1992); activation of human endothelial cells, Desch, C. E., et al., *Infect. Immun.*, Vol 57, 1612–4 (1992); and release of tumor necrosis factor (TNF) by human macrophages, Kuppermann, N., et al., *J. Infect. Dis.*, Vol 170, 630–5 (1994). Anti-LPS factor proteins also inhibit the growth of rough gram-negative bacteria. Morita, T., et al., *J. Biochem*, Vol. 97, 1611–20 (1985). In vivo, these proteins are protective against lethal *Escherichia coli* endotoxin challenge in rats, Wainwright, N. R., et al., supra.; and against lethal meningococcal challenge in rabbits, Alpert, G., et al., *J. Infect. Dis.*, Vol. 165, 494–500 (1992) or *E. coli*, Garcia, C. T., et al., *Crit. Care Med.* Vol 22:8, 1211–18 (1994). Recently, LALF was shown to improve survival in rabbits and rats with *E. coli* sepsis, Saladino, R., et al., *Circ. Shock*, Vol. 42, 104–10 (1994); Kuppermann, N., et al., *Pediatr. Res.*, Vol 31:32A (1992).

A rat model of endotoxemia caused by infection with an encapsulated strain of *E. coli* that is virulent in humans, was used to show that LALF at a concentration of 50 mg/kg has very potent anti-endotoxin activity in vivo. LALF blocked the lethal effects of endotoxin even after endotoxin had an opportunity to circulate and was likely to have bound to target cells. When compared with animals treated with the anti-endotoxin-monoclonal antibody HA-1A, animals treated with LALF showed significantly lower endotoxin concentrations and improved survival. HA-1A, while able to reduce circulating endotoxin concentrations, did not significantly improve survival. Kuppermann, N., et al., *J. Infect. Dis.*, Vol 170, 630–5 (1994).

Further, it has been shown that LALF given at a dose of either 2.5 or 5 mg/kg before lethal endotoxin challenge in rabbits, resulted in significant improvements in physiologic measurements and survival. LALF attenuated the toxic effects of *E. coli* endotoxin in rabbits and improved survival, even when administered after endotoxin challenge. Garcia, C., et al., *Crit. Care Med.*, Vol. 22:8, 1211–18 (1994). The administration of LALF 30 minutes after endotoxin challenge, however, had less protective activity than was obtained when it was administered before endotoxin challenge.

III. Antibiotics effective against gram-negative bacteria.

It has been discovered that anti-LPS factor proteins from horseshoe crabs potentiate, or produce a synergy, when combined with other antibiotics known to be effective against gram-negative bacterial infections. According to this invention, anti-LPS factor proteins can be used in combination with gram-negative antibiotics to produce an effective combination antibiotic for use in the treatment of gram-negative bacterial infections, endotoxemia and shock.

Gram-negative antimicrobials suitable for use in the claimed combination antibiotics include, but are not limited to, polymyxin B, ampicillin, amoxicillin, penicillin G, A tetracycline, erythromycin, spectinomycin, cefoxitin,

trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, minocycline, sulfonamides, nitrofurantoin, gentamicin, cefamandole, carbenicillin, ticarcillin, tobramycin, amikacin, A cephalosporin, cefoxitin, streptomycin, and clindamycin. Moreover, combinations may include more than one known gram-negative antibiotic and one or more anti-LPS factor proteins.

The discovery that anti-LPS factor proteins enhance the effectiveness of known antibiotics against gram-negative bacteria means that potent new antibiotics can be prepared using lower levels of each drug than would be necessary if either drug were administered alone. For illustration, polymyxin B (PMB) is a basic polypeptide antibiotic which has been shown to bind to, and structurally disrupt, the most toxic and biologically active component of endotoxin, Lipid A. PMB inhibits endotoxin activation of neutrophil granule release in vitro and is a potent treatment for gram-negative infections in humans. It has been shown that pretreatment with PMB is effective in preventing shock and mortality in rabbits receiving a potent dose of *E. coli* LPS. Baldwin, G., et al., *J. Infect. Dis.*, Vol. 164, 542–9 (1991). However, because of its systemic toxicity, this drug has limited use, except as a topical agent. WO 94/25476; WO 92/03535. According to this invention, a combination antibiotic comprising anti-LPS factor protein and polymyxin B, for example, can be prepared using lower concentrations of the potentially toxic polymyxin B due to the synergism produced by the inclusion of anti-LPS factor protein.

In another illustration, LALF may be combined with polymyxin B to treat septic shock caused by meningococcemia. Meningococcemia is the most common cause of septic shock in otherwise healthy children, and mortality from this condition remains high (10%) despite intensive therapy. Experiments with a meningococcal lipooligosaccharide-induced [LOS] septic shock model in rabbits, showed that pretreatment with polymyxin B alone failed to improve physiologic changes or mortality rate. Baldwin, G., et al., *J. Infect. Dis.*, Vol. 164, 542–9 (1991). By contrast, Limulus anti-LPS factor protein, LALF, significantly improved mean arterial pressure (MAP), arterial pH, serum bicarbonate concentrations, and survival even when administered 30 minutes after the lipooligosaccharide challenge. Alpert, G., *J. Infect. Dis.*, Vol. 165, 494–500 (1992). According to this invention, meningococcemia may be treated with the combination of LALF with polymyxin B.

It has been unexpectedly discovered that LALF effectively controls the growth of the gram-negative bacterium *Propionibacterium acnes*, which causes the common skin disease acne. Anti-LPS factor proteins can be used as antibiotics to treat acne either alone or in combination with other antibiotics such as tetracycline, or other medicinals such as benzoyl peroxide that are known to control *Propionibacterium acnes*. In the case of acne, anti-LPS factor proteins can be administered systemically, topically, or via simultaneous systemic and topical administration.

Therapeutic administration of the anti-LPS factor proteins alone, or in combination with gram-negative antibiotics, may be performed by methods known to those skilled in the art including topical, intravenous, intramuscular or subcutaneous routes, direct delivery into an infected body cavity by infusion, and oral or rectal administration.

A therapeutic dose of the claimed combination antibiotics is an amount that is effective to inhibit the growth of gram-negative bacteria and inhibit LPS-mediated stimulation of neutrophils and mononuclear cells. As used herein, inhibit means to inhibit at a level that is statistically significant and dose dependent. The terms "statistically significant" and "dose dependent" are well known to those skilled in the art.

In a preferred embodiment, a therapeutically effective amount of anti-LPS factor protein in the present invention is a concentration of between about 0.1 and 100 milligrams anti-LPS factor protein per kilogram of body weight. Other useful ranges include between about 0.1 and 1; 1 and 10; and 10 and 100 milligrams anti-LPS factor protein per kilogram body weight. A typical amount is between about 10 and 50 milligrams per kilogram body weight.

In addition, the amount of known gram-negative antibiotic included in the combination can be adjusted up or down based on known therapeutic doses and routine experimentation. The therapeutically effective amounts of the claimed combination antibiotics may be determined according to known methods based on the effective dosages discussed above.

IV. New antibiotics effective against gram-positive bacterial infections and new antimycotics effective against yeast infections comprising anti-LPS factor proteins.

Anti-LPS factor proteins effective against gram-positive bacteria.

Anti-LPS factor proteins have been discovered to be effective antibiotics against a wide variety of gram-positive bacteria. Thus, this invention is directed to pharmaceutical compositions containing anti-LPS factor proteins and the therapeutic use of these anti-LPS factor protein pharmaceutical compositions in the treatment of gram-positive bacterial infections. Further, anti-LPS factor proteins can be used in combination antibiotics known to be effective against gram-positive bacteria, including but not limited to penicillin G, erythromycin, A tetracycline, A cephalosporin, chloramphenicol, rifampin, aminoglycosides, vancomycin, and clindamycin. Pharmaceutical formulations or the administration of pharmaceutical compositions may include more than one anti-LPS factor protein and more than one antibiotic known to be effective against gram-positive bacteria.

A therapeutic dose of anti-LPS factor proteins either alone or in combination with known antibiotics to control gram-positive bacterial infection, is an amount that is effective to kill the gram-positive bacteria and control or inhibit the spread of the infection. An effective therapeutic dose can be determined by persons of ordinary skill in the art using known methods.

Anti-LPS factor proteins effective antimycotic agent.

It has also been discovered that anti-LPS factor proteins effectively inhibit the growth of yeast, for example, *Candida parapilosis* and *C. albicans*. It is therefore another embodiment of the present invention to use anti-LPS factor proteins as antimycotic agents either alone or in combination with other antimycotics, at a therapeutically effective dose to control yeast infections. There are many known antimycotics for treating yeast infections that can be administered in combination with anti-LPS factor proteins including but not limited to: amphotericin B, clotrimazole, flucytosine, grisefulvin, haloprogin, hydroxystilbamidine, miconazole, nystatin, and tolnaftate. Combinations may be made of anti-LPS factor proteins and more than one other antimycotic agent.

As used herein, the therapeutic dose of the claimed anti-LPS factor protein-containing antibiotics or antimycotics is an amount that is effective to inhibit the growth of gram-positive bacteria or yeast. In an embodiment, a therapeutically effective amount of anti-LPS factor protein is a concentration of between about 0.1 and 100 milligrams anti-LPS factor protein per kilogram of body weight. Other useful ranges include between about 0.1 and 1; 1 and 10; and 10 and 100 milligrams anti-LPS factor protein per kilogram body weight. A typical amount is between about 10 and 50 milligrams per kilogram body weight.

The amount of known antibiotic or antimycotic agent included in the combination formulation can be adjusted up or down based on known therapeutic doses and routine experimentation. The therapeutically effective amounts of the claimed antibiotics and antimycotics may be determined according to known methods based on the effective dosages given above.

V. Preparation of Pharmaceutical Compositions.

The pharmaceutical compositions containing anti-LPS factor proteins of the present invention can be administered either alone or in combination with other known drugs in vivo in a pharmaceutically or veterinarilly acceptable carrier. If necessary, an adjuvant to facilitate absorbtion may be included in the formulation.

The term "carrier" as used herein means a synthetic or natural, inorganic or organic substance which is added to the endotoxin binding protein of the present invention to assist the active ingredient in reaching the location to be treated therewith and to facilitate storage, transportation and handling of the active ingredient.

Among suitable liquid carriers, there may be included aromatic hydrocarbons such as benzene, toluene, and xylene; paraffinic hydrocarbons such as mineral oil and the like; halogenated hydrocarbons such as carbon tetrachloride, chloroform, dichlorocthane and the like; ketones such as acetone, methyl ethyl ketone, etc.; ethers such as dioxane, tetrahydrofuran and the like; alcohols such as methanol, propanol, ethylene glycol and the like; or dimethyl formamide, dimethylsulfoxide, water, etc. Mixtures of any number of liquid carriers are also envisioned. Dissolution of lyophilized anti-LPS factor proteins in unbuffered pyrogen-free distilled water or saline or phosphate buffered saline, can be achieved by adjusting the pH until the solution becomes water clear. For this reason, the preferred liquid carrier is pyrogen-free distilled water or saline adjusted to the appropriate pH to facilitate solubility of the anti-LPS factor proteins.

In order to enhance the effectiveness of the compound according to this invention, it is possible to use such adjuvants as given below, either singly or in combination, in accordance with the purpose of each application thereof while taking into consideration the form of their preparation and their field of application.

Exemplary adjuvants may include anionic surfactants such as alkyl sulfates, aryl sulfonates, succinates, polyethylene glycol, alkyl ether sulfates, and the like; cationic surfactants such as alkylamines, polyoxyethylene alkylamines, etc.; non-ionic surfactants such as polyoxyethylene glycol ethers, polyoxyethylene glycol esters, polyol esters and the like; and amphoteric surfactants. Encapsulation or microencapsulation of the active ingredient in liposome vesicles is also within the scope of this invention.

Examples of stabilizers, thickeners, lubricants and the like are isopropyl hydrogen-phosphate, calcium stearate, wax, casein, sodium alginate, serum albumin, other blood proteins, methylcellulose, carboxymethylcellulose, gum arabic, etc. It should be kept in mind that these ingredients are not limited to the recited examples.

Solutions or suspensions containing anti-LPS factor proteins may also include the following components: a sterile diluent such as water for injection, saline solution, oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple base vials made of glass or plastic.

Specific indications or diseases that may be treated by the anti-LPS factor proteins, or combination antibiotics that include anti-LPS factor proteins, include but are not limited to acne, septicemia, toxic shock, gram-negative bacterial infections, endotoxin-related arthritis, gonorrhea, periodontal disease, spinal meningitis, infections of amniotic fluid, gram-positive bacterial infections, yeast infections, and mold growth.

VI. Preparation of pharmaceutical compositions suitable for topical use comprising anti-LPS factor proteins.

In addition to systemic administration, it is also within the scope of this invention to formulate anti-LPS factor proteins into pharmaceutical compositions suitable for topical use to promote wound healing or to treat vaginal yeast infections. Topically applied anti-LPS factor proteins, either alone or in combination with other antimicrobials, can prevent or control gram-negative and gram-positive bacterial infections, yeast infections and the growth of mold.

A preferred embodiment includes topical formulations of anti-LPS factor protein alone or in combination with known antibiotics or antimycotics, suitable for application to incisions or exposed tissue for the promotion of wound healing by curing or preventing bacterial or yeast infections. In another embodiment, anti-LPS factor proteins are formulated into suppositories to treat vaginal yeast infections.

There are no limitations as to the type of wound or other traumata that can be treated, and these include: first, second and third degree burns, especially second and third degree; epidermal and internal surgical incisions, including those of cosmetic surgery; wounds, including lacerations, incisions, and penetrations; and epidermal ulcers including decubital (bed sores), diabetic, dental, hemophiliac, and varicose.

Anti-LPS factor protein compositions are applied to burns in the form of a sterile irrigant, preferably in combination with a physiological saline solution, or in the form of ointments or suspensions, preferably in combination with purified collagen. The compositions may also be impregnated into transdermal patches, plasters, bandages, or sterile implants preferably in a liquid or semi-liquid form.

A therapeutically effective dose of anti-LPS factor protein is a dose that inhibits the growth of bacteria, yeast, and mold when applied topically. The range of acceptable doses of anti-LPS factor proteins for topical application includes between about 0.01 and 10 weight percent. Where known antibiotic or antimycotic agents are combined with anti-LPS factor proteins, their concentration can be varied up or down based on the range of known clinically acceptable concentrations for these drugs.

Initial dosing of anti-LPS factor protein either alone or in combination with other agents, should be delivered topically to the therapeutic site at a concentration of about 0.5 weight percent. This dose can be thereafter adjusted up or down in line with clinical experience. Continued application or periodic reapplication of the compositions is indicated. The clinician will be expected to modify the dosage in accordance with clinical experience.

In a preferred embodiment, anti-LPS factor proteins are topically applied at a concentration of between about 0.01 and 10 weight percent in a pharmaceutically acceptable carrier. Other preferred embodiments include application at concentrations of between about 0.1 and 1; 1 and 2; 2 and 5; 5 and 7; and 7 and 10 weight percent; most preferred is between about 1 and 2 weight percent.

VII. Use of anti-LPS factor proteins as an antimicrobial preservative either alone or in combination with conventional preservatives.

Endotoxin is shed from living bacteria and is also released into the environment when bacteria die and decompose.

Since gram-negative bacteria are found in great numbers in air, water, and soil, bacterial endotoxin commonly contaminates raw materials and processing equipment used in the manufacturing of cosmetics and skin or hair preparations. Contamination by gram-positive bacteria, yeast and mold is also common.

This invention is also directed to a new preservative for use in topically applied cosmetics and skin or hair preparations that inhibits and controls the growth of a wide variety of microbial contaminants.

Preservatives are typically employed in cosmetics and skin and hair preparations because they are usually manufactured under clean, but non-sterile conditions. The preservatives are used to prevent the growth of microbes including bacteria, yeast and mold. A sufficient quantity of one or more preservatives is typically added so that the cosmetic or preparation resists the growth of bacteria from an experimental inoculation for extended periods of time.

There is a need for new, effective preservatives in the cosmetic industry. Many consumers have been sensitized to currently available preservatives and have developed allergic reactions. It has been discovered that relatively low doses of anti-LPS factor proteins act as preservatives by preventing or suppressing the growth of a broad range of gram-negative and gram-positive bacteria as well as some yeasts and mold, for extended periods of time.

Anti-LPS factor proteins can be used alone or in combination with known preservatives in topically applied cosmetics, or skin or hair preparations. The cosmetics and skin or hair preparations may be powders, creams, lotions, or gels. Some preservatives that are typically used are imidazolidinyl urea, sodium hydroxy methylglycinate, diazolidinyl urea, glyoxyl diureide, chlorphenesin, methylparaben, an ester of p-hydroxy-benzoic acid, chloromethyl-thiazoline, methyl-isothiazoline, phenyoxyethanol, hexetidine, chloro-hexydingluconate, and the parabens: butyl, isobutyl, methyl, propyl, and isopropyl. Another commercially available preservative that may be used in combination with anti-LPS factor protein is known as PHENONIP™ which is a practically colorless, viscous, liquid mixture of phenoxyethanol, methylparaben, ethylparaben, propylparaben, and butylparaben available from Nipa Laboratories, Inc., Wilmington, Del.

The anti-LPS factor proteins appear to act as potentiators of preservatives used in cosmetics and skin and hair preparations. By using anti-LPS factor protein as a preservative in a cosmetic or a skin or hair preparation, the concentration of previously known preservatives may be reduced or eliminated. Thus, anti-LPS factor proteins not only act as preservatives, they also minimize the antigenicity of the cosmetic or lotion by replacing commonly used preservatives to which some consumers have become sensitized. Anti-LPS factor proteins also neutralize endotoxin which is another source of antigenicity.

The amount of anti-LPS factor protein that may be used as a preservative varies from between about 0.005 and 5 weight percent of the cosmetic or lotion. It is preferred that the lowest effective amount of anti-LPS factor protein be used to prevent sensitization of the user to anti-LPS factor protein. This amount can be determined by routine experimentation and it will vary according to whether anti-LPS factor protein is used alone or in combination with other preservatives. The amount used will also depend upon the formulation of the cosmetic or lotion and the storage conditions.

In a preferred embodiment, anti-LPS factor protein is present as a preservative at a concentration of between about 0.005 and 0.01; 0.01 and 0.1; 0.1 and 1; 1 and 2; or 2 and 5 weight percent with the most preferred being between about 0.01 and 1 weight percent.

Various aspects of the invention are illustrated in the following examples to aid in understanding the invention, which is not intended to be limiting.

EXAMPLES

Example 1: Expression and Purification of Recombinant LALF in Pichia

Fermentation: LALF protein expression in Pichia:

The gene encoding LALF is shown in FIG. 1, SEQ ID NO:1. The LALF gene has been cloned into the Pichia pPIC9K vector as an in-frame fusion with the alpha mating factor secretion signal. The gene encoding LALF (SEQ ID NO:1) was synthesized by Genosys, Inc. The design of the synthetic gene was based on the sequence of the deduced amino acid sequence from the native LALF protein sequence. Codons were chosen which optimize expression in yeast (based on *Saccharomyces cerevisiae*). The amino terminal sequence, N-Asp-Gly-Ile-Trp-Thr, is ideally suited for correct cleavage of the yeast alpha mating factor secretion signal. The gene was modified using PCR mutagenesis to incorporate 5' cloning sites that juxtapose the codon for the N-terminal Asp residue found in the mature LALF with the codon for the last amino acid residue in the yeast alpha mating factor secretion signal. Kex2-like protease cleavage of the fusion protein generates a secreted, mature, native form of the LALF protein.

Expression is driven by the Pichia methanol-inducible AOX1 promoter. The *Saccharomyces cerevisiae* a-factor prepro leader peptide, present on the expressed protein as an N-terminal fusion, targets the protein for secretion into the media and is cleaved off in the process. Expression of the protein of interest is induced by growing the His+ recombinant strain in methanol-containing media. Scorer, C. A., et al, *Gene*, Vol. 136, 111–19 (1993); Scorer, C. A., et al., *BioTechnology*, Vol. 12, 181–4 (1994).

Filtration: The media containing the secreted protein, is clarified by hollow fiber diafiltration (0.45 micron, A/G Technologies) at high flow rate (1–5 liters/min.) and low back pressure (5–10 psi).

Ultrafiltration: The first purification step is achieved by collecting the filtrate from a 30,000 Dalton cut-off tangential flow ultrafiltration membrane cassette. This filtrate is concentrated by a 8,000 dalton cut-off membrane, achieving a rapid size exclusion.

Chromatography: Concentrated 8–30,000 dalton crude LALF is loaded onto a cation exchange column. After extensive washing with Phosphate Buffered Saline (PBS), a linear gradient of NaCl, 0-1 Molar in PBS is used to elute the purified LALF. The eluate may be desalted by ultrafiltration, or if necessary, a second step purification by reversed-phase chromatography may be performed. The high salt peak is loaded directly on the column and eluted with a linear gradient of 0–50% isopropanol, 0.1% Trifluoroacetic acid. It is preferable to freeze, lyophilize and store the purified LALF at 20° C. until used.

Example 2: The Combination of LALF with Polymyxin B Produces a Synergistic Effect Against Gram-negative Bacteria

E. coli cells were cultured overnight at 37° C. in Difco Luria Broth (LB). Aliquots were diluted 1:100 in fresh LB (control). LB plus polymyxin B (PMB) was serially diluted from 0.25 micrograms/ml to 0.001 micrograms/ml. LB plus LALF was formulated at concentrations from 20 to 0.07 micrograms/ml. Combinations of polymyxin B and LALF were formulated as indicated in FIG. 2. Optical density was recorded at 410 nm during the course of the experiment.

FIG. 2A shows the inhibitory effect of polymyxin B by itself on *E. coli* at concentrations ranging from 0.001 to 0.25 micrograms/ml. As one dilutes the antibiotic, the growth of the *E. coli*, as measured by optical density, increases. A

concentration of 0.06 micrograms PMB/ml was chosen for the subsequent LALF combination experiments shown in FIG. 2B because 0.06 micrograms PMB is only slightly effective (approximately 20% inhibition of growth) against *E. coli*.

FIG. 2B shows that the addition of LALF to PMB produces a synergistic antibiotic effect on the growth of gram-negative bacteria. The concentration of PMB is held constant at 0.06 micrograms/ml throughout the experiment. The level of growth of *E. coli* allowed by 0.06 micrograms/ml PMB alone is shown by the dashed line. The antibiotic effect of LALF alone at concentrations ranging from 20 to 0.07 micrograms/ml, is indicated by the solid black bars.

To test the potentiation of the antibiotic effect of PMB by LALF, a combination antibiotic was formed comprising 0.06 micrograms/ml polymyxin B and various concentrations of LALF ranging from 20 to 0.07 micrograms/ml. The level of growth of *E. coli* in the combined antibiotic PMB/LALF is indicated by the cross-hatched bars. The results show that LALF across a broad range of concentrations produces a synergistic effect when combined with 0.06 micrograms/ml PMB.

Example 3: The Combination of LALF with Ampicillin Produces a Synergistic Effect Against Gram-negative Bacteria

It has also been discovered that LALF produces an in vitro synergism in controlling gram-negative bacteria when combined with the antibiotic ampicillin, a well known semisynthetic, acid-resistant form of penicillin.

E. coli cells were cultured overnight at 37° C. in Difco Luria Broth (LB). Aliquots were diluted 1:100 in fresh LB (control). Test samples consisted of LB plus ampicillin at 2.5 micrograms/ml; LB plus LALF at 2.5 and 5 micrograms/ml; and combinations of 2.5 micrograms/ml ampicillin and either 2.5 or 5.0 micrograms/ml LALF. Optical density was recorded at 410 nm during the course of the experiment.

The combination of 2.5 micrograms/ml LALF and 2.5 micrograms/ml ampicillin, produced a synergistic effect in inhibiting the growth of *E. coli*.

Example 4: LALF is an Effective Antibiotic Against *Propionibacterium acnes*

The antibiotic effect of recombinant LALF was tested against *Propionibacterium acnes*, the bacterium that causes acne, using the zone inhibition method. Petri dishes were filled with sterile nutrient agar following standard microbiological procedures. The nutrient agar-filled dishes were allowed to cool and harden before being inoculated with test bacteria. Using a sterile swab, the surface of the petri dishes were inoculated with a culture of *Propionibacterium acnes*, ATCC No. 11827, that had grown overnight at 37° C.

Sterile filter paper discs (4 mm, Whatman) were treated with 10 microliters of aqueous solutions of (1) recombinant LALF at a concentration of 1 mg/ml: the test sample; (2) benzoyl peroxide at a concentration of 0.05%: the positive control; or (3) sterile saline: the negative control. Benzoyl peroxide serves as a positive control because it is known to inhibit the growth of *Propionibacterium acnes*.

The treated filter paper discs were incubated overnight at 37° C. Incubation in LALF produced a zone of inhibition around the test sample having a diameter of 4–5 mm. Incubation in benzoyl peroxide (the positive control) produced a zone of inhibition having a diameter of 10–11 mm. The negative control treated with sterile saline had no zone of inhibition. These results show that recombinant LALF is an effective antibiotic against *Propionibacterium acnes*.

Example 5: LALF is an Effective Antibiotic Against Gram-Positive Bacteria

Uncharacterized gram-positive bacteria isolated from marine organisms, were cultured overnight at 37° C. in Difco Luria Broth (LB). Aliquots were diluted 1:100 in fresh LB (control). Test samples consisted of LB plus LALF at 2.5 and 5.0 micrograms/ml. Optical density was recorded at 410 nm during the course of the experiment to monitor the growth of gram-positive bacteria.

FIG. 4 shows that LALF at concentrations of either 2.5 or 5 micrograms/ml is effective at inhibiting the growth of gram-positive bacteria over the five hour course of the experiment.

Example 6: LALF is an Effective Antimicrobial Preservative in Topically Applied Cosmetics and Lotions Against a Broad Spectrum of Microbes

Challenge microorganisms were prepared as pools of related organisms which were grown in the appropriate medium. The cell number was quantitated by serial dilution:

TABLE I

Pool 1	Pool 2	Pool 3
(Gram-negative)	(Gram-negative)	(Gram-positive)
Escherischia coli	*Pseudomonas cepacia*	*Staph. epidermidis*
Enterobacter gergoriae	*P. putida*	*Staph. aeureus*
E agglomerans	*P. stutzeri*	
Klebsiella pneumoniae	*P. aeruginosa*	
Pool 4	**Pool 5**	
(Yeast)	(Mold)	
Candida parapilosis	*Aspergillus niger*	
C. albicans		

An oil in water emulsion was prepared comprised of the following ingredients:

TABLE II

Cetyl alcohol (fatty alcohol)	1.65 g
Glycerol stearate	1.65
Ariacel 165 (surfactant)	6.60
Debydag wax (surfactant)	1.10
Softisan 378 (triglyceride/oil)	.50
Silicone 200/100	.40
Cetiol LC	3.60
Tween 40 (emulsifier)	.66
Ariacel 40	.44
DI Water (distilled)	74.00
1.3 Btgly	6.00
EDTA (chelator)	.10

For control experiments, the formula was prepared as above. To test anti-LPS antibiotic activity, LALF was added to the emulsion to achieve a concentration of 0.01 weight percent, with appropriate adjustment of the DI Water.

Control and anti-LPS emulsions were spiked with 10^6 viable cells from each of the five microorganism pools. Cell number was quantitated by serial dilution. The number of viable microbial cells was determined by plate count at the time of the initial spike, and at weekly intervals up to 8 weeks. The number of viable cells was determined by taking an aliquot from the sample, diluting it and culturing it so that colonies arising from individual cells could be counted. After obtaining an aliquot for testing at Week 3, microbes were re-spiked to initial levels as indicated in Table III.

TABLE III

LALF Emulsion

Pools	(spike) Initial	Wk 1	Wk 2	(spike) Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8
Results:									
1	6.0	<1.0	<1.0	<1.0	2.7	<1.0	<1.0	<1.0	<1.0
2	6.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
3	6.0	3.2	<1.0	<1.0	3.0	<1.0	<1.0	<1.0	<1.0
4	6.0	<1.0	<1.0	<1.0	3.0	<1.0	<1.0	1.7	<1.0
5	5.0	5.0	3.0	2.6	2.8	2.7	<1.0	3.0	2.7
Control:									
1	6.0	6.0	3.0	<1.0	6.0	6.0		6.0	6.0
2	6.0	6.0	2.0	<1.0	6.0	6.0		6.0	6.0
3	6.0	6.0	2.3	2.6	6.0	6.0		6.0	6.0
4	6.0	6.0	1.7	<1.0	6.0	6.0		6.0	6.0
5	5.0	5.0	5.0	5.0	5.0	5.0		5.0	5.0

The results of this example show that LALF is an effective antimicrobial preservative in topically applied cosmetics and lotions. LALF at a relatively low concentration of 0.01 percent (weight/volume), was able to kill a wide spectrum of bacteria and prevent their regrowth over an eight week period, even when the emulsion was re-spiked with bacteria at three weeks. LALF was also effective at killing and preventing the regrowth of the yeasts *Candida parapilosis* and *albicans*. While LALF was less effective against the mold *Aspergillus niger* than against bacteria and yeast, it nonetheless limited the growth of mold. LALF therefore acted as a mycostatic agent with respect to mold while it more ressively controls the growth of bacteria and yeast.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious to one of skill in the art that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 303 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GATGGTATTT GGACTCAATT GATTTTTACT TTGGTTAATA ATTTGGCTAC TTTGTGGCAA   60
TCTGGTGATT TTCAATTTTT GGATCATGAA TGTCATTATA GAATTAAACC AACTTTTAGA  120
AGATTGAAAT GGAAATATAA AGGTAAATTT TGGTGTCCAT CTTGGACTTC TATTACTGGT  180
AGAGCTACTA AATCTTCTAG ATCTGGTGCT GTTGAACATT CTGTTAGAAA TTTTGTTGGT  240
CAAGCTAAAT CTTCTGGTTT GATTACTCAA AGACAAGCTG AACAATTTAT TTCTCAATAT  300
AAT                                                                303
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

-continued

(2) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp	Gly	Ile	Trp	Thr	Gln	Leu	Ile	Phe	Thr	Leu	Val	Asn	Asn	Leu
				5					10					15
Ala	Thr	Leu	Trp	Gln	Ser	Gly	Asp	Phe	Gln	Phe	Leu	Asp	His	Glu
				20					25					30
Cys	His	Tyr	Arg	Ile	Lys	Pro	Thr	Phe	Arg	Arg	Leu	Lys	Trp	Lys
				35					40					45
Tyr	Lys	Gly	Lys	Phe	Trp	Cys	Pro	Ser	Trp	Thr	Ser	Ile	Thr	Gly
				50					55					60
Arg	Ala	Thr	Lys	Ser	Ser	Arg	Ser	Gly	Ala	Val	Glu	His	Ser	Val
				65					70					75
Arg	Asn	Phe	Val	Gly	Gln	Ala	Lys	Ser	Ser	Gly	Leu	Ile	Thr	Gln
				80					85					90
Arg	Gln	Ala	Glu	Gln	Phe	Ile	Ser	Gln	Tyr	Asn				
				95					100					

What is claimed is:

1. A preservative for use in a topically applied cosmetic or skin or hair preparation, comprising one or more anti-LPS factor proteins, wherein said anti-LPS factor protein is present in an amount sufficient to inhibit the growth of bacteria, yeast and mold.

2. The preservative according to claim 1 further comprising a commercially available preservative.

3. The preservative according to claim 2 wherein the commercially available preservative is selected from the group consisting of imidazolidinyl urea, sodium hydroxy methylglycinate, diazolidinyl urea, glyoxyl diurcide, chlorphenesin, methylparaben, an ester of p-hydroxybenzoic acid, chloro-methyl-thiazoline, methyl isothiazoline, phenyoxyethanol, hexetidine, chlorohexydingluconate, butylparaben, isobutylparaben, methylparaben, propylparaben, and isopropylparaben.

4. The method of preserving a cosmetic or skin or hair preparation, comprising adding an effective amount of one or more anti-LPS factor proteins as a broad spectrum antimicrobial preservative, either alone or in combination with one or more preservatives.

5. A method of minimizing the antigencity of a cosmetic or skin or hair preparation comprising replacing a sensitive commercially available preservative with one or more anti-LPS factor proteins.

6. The method of claim 5 wherein the anti-LPS factor protein neutralizes endotoxin.

* * * * *

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Associates of Cape Cod, Inc.
704 Main Street
Falmouth, Massachusetts 02540

Gentlemen:

As a condition of my employment with you, and in consideration for the compensation and other benefits I receive from you, I hereby agree as follows:

1. Except as hereinafter provided in paragraph 7. below, any and all papers, reports or studies that I may write or to which I may in any way contribute, resulting in whole or in part from research in which I engage as your employee, shall be your sole property and you shall have the exclusive right to publish or not to publish, in whatever form, the results of such research;

2. I hereby grant to you all of my rights to patents, copyrights, royalties (exclusive of the following patents applied for or to be applied for from work preformed during my last employment; a. Hybrid Nucleic Acid Sequences Having Tandemly Arranged Genes, co-inventor: Nancy Lee, Serial #577,291, Filed February 6, 1984; b. Nuclease and Protease Inhibitors Isolated from Occular Lens Tissue; c. Plasminogen Activator Isolated from a Soil Organism; along with royalties mentioned in paragraph 7. below) or other benefits of whatever nature resulting from research in which I engage as your employee and will assist you at all times in every proper way (but only at your expense) to obtain for your benefit such patents, copyrights, royalties, benefits or other forms of protection therefor;

3. I shall disclose fully to the President of this Company any and all inventions, discoveries, research projects, and scientific papers which I shall conceive, engage in, or write as a result of research in which I engage as your employee.

4. Except as required by law or as authorized by a Company officer, I will not at any time reveal, divulge or make known to anyone any information or knowledge which I received or acquire as a results of research in which I engage as your employee concerning.

(a) the method, process, or manner of producing any product of this Company;

(b) any methods or devices used by the Company in its business; or

(c) any secret or confidential information concerning the Company or its business.

5. I further agree that I shall not solicit, interfere with, or endeavor to entrice away from the Company any customer of the Company or any person, firm, or corporation in the habit of dealing with the Company, or any employee of the Company.

6. In the event of termination of my employment by either party, I will not for a period of two years after such termination, own, manage, operate, control, be employed by, participate in or perform services for any business, whether incorporated or unincorporated, or in partnership form, which competes with the Company.

7. I hereby reserve the right of ownership of all papers, reference books, text books, speeches, and course materials which I may write, publish or present in any capacity other than as your employee, and I hereby also reserve the right to receive for my own account royalties, honoraria, and fees for such property.

8. The Company and I understand that my employment is terminable at will, that is, it can be terminated by me or by the Company at any time without either party having to show sufficient cause.

ASSOCIATES OF CAPE COD, INC.

By: Stanley D Watson

Signature of Employee

Dated: 6/6/86